PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/655, 7/08, A61K 38/31, 38/10

A2

(11) International Publication Number: WO 98/09991

(43) International Publication Date: 12 March 1998 (12.03.98)

(21) International Application Number: PCT/US97/15578

(22) International Filing Date: 4 September 1997 (04.09.97)

(30) Priority Data:
60/025,468 5 September 1996 (05.09.96) US

(71) Applicants: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). UNIVERSITY OF WASHINGTON [US/US]; Seattle, WA 98195 (US).

(72) Inventors: ENSINCK, John, W.; 232 Dorffel Drive East, Seattle, WA 98112 (US). VOGEL, Robin, E.; 9230 S.W. 192nd Street, Vashon, WA 98070 (US). LASCHANSKY, Ellen, C.; 2229 11th Avenue East, Seattle, WA 98102 (US). FRANCIS, Bruce, H.; 1660 Interlaken Place East, Seattle, WA 98112 (US). BASKIN, Denis, G.; 6205 145th Avenue N.E., Redmond, WA 98052 (US). D'ALESSIO, David, A.; 4607 Woodland Park Avenue North, Seattle, WA 98103 (US). HOFFMAN, Ross, C.; Apartment 302, 503 13th Avenue East, Seattle, WA 98102 (US).

(74) Agent: LEITH, Debra, K.; ZymoGenetics, Inc., Director, Intellectual Property & Licensing, 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ENTERIC NEURAL PEPTIDE

(57) Abstract

The present invention relates to an intestinal peptide that is located in mammalian distal ileum. The peptide, designated "enteric neural peptide" or "ENP", corresponds to the N-terminal, 13 amino acid fragment of somatostatin-28. ENP is localized in cells that do not express S-28 and do not contain the C-terminal S-14 fragment of prosomatostatin. ENP increases in the circulation within 30 minutes of intake of mixed food, wherein the predominant nutrient signal is fat. Antibodies that bind ENP are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Description

ENTERIC NEURAL PEPTIDE

BACKGROUND OF THE INVENTION

When mammals consume food, satiety signals are generated that tell the mammal to stop eating. Likewise, after periods of fasting, "hunger" signals tell the mammal to eat. Some signals may act directly on gastrointestinal (enteric) tissue; others may involve neural components. Because the gastrointestinal cells exhibit rapid turnover, molecules produced locally, such as peptide growth factors, modulate intestinal epithelial growth. These peptide growth factors include epidermal growth factor, transforming growth factor α, and insulin-like growth factor 1. Peptide hormones, such as neurotensin, cholecystokinin (CCK), bombesin and peptide YY, also play a role in intestinal epithelial proliferation. Proglucagon-derived peptides, such as glicentin, GLP-1 and GLP-2, are synthesized by intestinal tissues and may affect enteric epithelial cell growth. Several of these peptides, including GLP-1 and CCK, have also been reported to be involved in biological mechanisms controlling obesity, feeding behavior, and insulin secretion. Peptides that are secreted by gastrointestinal tissue, but that act on or interact with neural tissues or pathways, are referred to as "brain-gut peptides", "gut-brain peptides" or "enteric-neural peptides."

An exemplary enteric-neural peptide is glucagon-like peptide 1 (GLP-1; 7-36amide$_2$), an insulinotropic peptide cleaved from proglucagon in epithelial endocrine cells in the distal intestine (D. D'Alessio et al., J. Clin. Invest. 93:2263-66, 1994). Ingestion of glucose or a mixed meal stimulates release of GLP-1 into the

circulation, and plasma levels of GLP-1 rise concurrently with insulin and glucose. GLP-1 decreases post-prandial glucose plasma levels by increasing insulin secretion, lowering glucagon release, and stimulating insulin-independent glucose disposal in peripheral tissues. Exogenous GLP-1 inhibits gastric emptying of liquid meals. GLP-1 and its specific receptors are also present in the hypothalamus, and intracerebroventricular (ICV) injection of GLP-1 has been reported to inhibit feeding in fasted rats (M.D. Turton et al., Nature 379:69-72, 1996).

Another exemplary gut-brain peptide that has been associated with fat ingestion is galanin, a 29-30 amino acid neuropeptide. Galanin has multiple biological effects in both the central and peripheral nervous systems (K. Bedecs et al., Intl. J. Biochem. Cell Biol. 27:337-49, 1995).. For instance, galanin inhibits glucose-induced insulin release and hippocampal acetylcholine release; stimulates food (fat) intake and growth hormone release; and causes contraction of the jejunum. Galanin is found in high concentrations within the hypothalamus. More particularly, a close positive association between galanin in the paraventricular nucleus (PVN), particularly its midlateral region, and fat ingestion has been reported (A. Akabayashi et al., Proc. Natl. Acad. Sci. USA 91:10375-59, 1994). No relationship between galanin and other brain areas, or PVN-galanin and carbohydrate and protein ingestion, was observed. PVN injection of antisense oligonucleotides corresponding to galanin mRNA resulted in a decrease in fat ingestion and body weight. In contrast, neuropeptide Y (NPY) and norepinephrine stimulate carbohydrate ingestion after PVN injection. Further, NPY levels decrease in rats fed a high carbohydrate diet, indicating a negative feedback mechanism; galanin levels increase in rats fed a high fat diet, indicating a positive feedback mechanism. In the anterior pituitary, galanin mRNA levels and immunoreactivity are higher in

females than in males, and these levels vary with the estrous cycle, decrease with ovariectomy, and increase after estradiol treatment. Galanin levels also rise just after puberty in females, when estrogen production and fat intake are simultaneously increasing (A. Akabayashi, Japan. J. Psychosomatic Med. 36:97-105, 1996). Galanin may be implicated in binge eating or other eating disorders.

The galanin receptor is a Gi-protein-coupled, membrane-bound glycoprotein with an estimated molecular weight of 53 kDa. The N-terminal first 16 amino acid residues are necessary and sufficient for receptor recognition and receptor activation (K. Bedecs et al., *supra*). Galanin is involved in effector systems, such as K^+/Ca^{2+}-channels and adenylate cyclase (T. Bartfai et al., Crit. Rev. Neurobiol. 7:229-74, 1993). The distribution of galanin receptors and of galanin-like immunoreactivity are overlapping in the CNS. Synthetic, high affinity galanin receptor antagonists that are peptides have been developed. These antagonists are useful tools in analyzing the function(s) of endogenous galanin, and permit further characterization and subtyping of galanin receptors.

The present invention provides an enteric neural peptide that may find use in treatment of satiety/obesity, eating disorders or diabetes. In addition, the enteric neural peptide may be used as a peptide growth factor or a modulator of intestinal function. These and other uses of the enteric neural peptide should be apparent to those skilled in the art from the teachings herein.

SUMMARY OF THE INVENTION

Within one aspect, the present invention provides a method for regulating obesity or an eating disorder in a mammal, comprising: administering to a mammal in need thereof a composition comprising a

mammalian polypeptide having 90% amino acid sequence identity to SEQ ID NO. 2 in combination with a pharmaceutically acceptable vehicle in an amount sufficient to regulate obesity or the eating disorder. A preferred polypeptide for this purpose has the amino acid sequence SANSNPAMAPRER.

Within a second aspect of the invention there is provided a method for treating diabetes in a mammal, comprising: administering to a mammal in need thereof a composition comprising a mammalian polypeptide having 90% amino acid sequence identity to SEQ ID NO. 2 in combination with a pharmaceutically acceptable vehicle in an amount sufficient to treat diabetes.

Within a third aspect of the invention there is provided an antibody that binds to Asn(5)-Pro(6)-Ala(7) of prosomatostatin.

DETAILED DESCRIPTION OF THE INVENTION

The term "ortholog" (or "species homolog") denotes a polypeptide or protein obtained from one species that has homology to an analogous polypeptide or protein from a different species.

The term "paralog" denotes a polypeptide or protein obtained from a given species that has homology to a distinct polypeptide or protein from that same species.

The term "allelic variant" denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The term "expression vector" denotes a DNA molecule, linear or circular, that comprises a segment

encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide molecule, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985). When applied to a protein, the term "isolated" indicates that the protein is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the protein in a highly purified form, i.e., greater than 95% pure, more preferably greater than 99% pure.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

The term "complements of polynucleotide molecules" denotes polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "promoter" denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger peptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-

binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. Most nuclear receptors also exhibit a multi-domain structure, including an amino-terminal, transactivating domain, a DNA binding domain and a ligand binding domain. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^9$ M^{-1}.

The present invention is based in part upon the discovery of a novel intestinal peptide that is located in neurons of distal mammalian gut and is secreted into the

circulation in a regulated manner. The plasma levels of this peptide increased 2-3 fold within 30 min after intake of mixed food, with fat being the predominant nutrient signal. Antiserum that reacted with this novel intestinal peptide did not cross-react with other enteric neural peptides. The novel peptide has been localized to the mammalian distal ileum, and more specifically to neuron cell bodies within the myenteric plexus and neuronal axons ramifying into the ileum mucosa. The novel peptide has an apparent molecular weight of 3500 Da, as determined by gel filtration in 6 M guanidine, and has been designated "5K" or "Enteric Neural Peptide (ENP)."

ENP was initially identified using polyclonal antisera raised to synthetic peptides that span different regions of prosomatostatin. Prosomatostatin (S-28) is processed from S-28 to a C-terminal, cyclic, 14 amino acid fragment (referred to in the literature as somatostatin-14 or S-14). This C-terminal S-14 molecule inhibits growth hormone release. Residues 7-10 of S-14 have been shown to be essential for the biological activity of somatostatin (The Pharmacological Basis of Therapeutics, 9th Ed., Goodman & Gilman, 1996, Chapter 55, pp. 1369-70). Somatostatin has also been reported to inhibit secretion of insulin and glucagon, and has been identified in pancreatic islet cells.

Based on immunological reactivity and cross-reactivity, however, ENP did not appear to be derived from prosomatostatin. Instead, ENP's tissue localization in the intestine, and its rapid release into the circulation following fat intake, suggested that ENP was a novel enteric neural (gut-brain) peptide that can act as a hormone.

Partial purification of ENP was subsequently performed. Amino acid sequencing and MALDI analysis of semi-pure fractions containing ENP indicated that ENP corresponds to the first 13 residues of somatostatin-28

(i.e, N-terminal residues 1-13; also designated NT 1-13 or S-28[1-13]). MALDI data showed a mass consistent with the mass of NT 1-13 having an oxidized Met (1416 Da). Synthetic NT 1-13 displayed a higher affinity than S-28 for the F4 polyclonal antiserum. Generally, complete processing of somatostatin produces an N-terminal 14 amino acid fragment that is converted to a 12 amino acid fragment (S-28[1-12]) after some protease clipping. S-28 is the predominant form in the gut; the C-terminal S-14 is the predominant form in the brain. At present, no biological function has been attributed to the NT 1-13 fragment of S-28.

ENP has been localized in tissues and cells where S-28 does not appear (i.e., mucosal/ endocrine cells in the small intestine). Further, S-28 is not found associated with gastrointestinal nerves. NT 1-13 has been found in the distal gut of baboons, where prosomatostatin is not expressed and S-14 is not found. Immunocytochemistry is performed to determine what cells in the distal gut are positive for NT 1-13, but negative for S-28. Also, purified, synthetic NT 1-13 is injected into rats, and their feeding behavior is observed. Purified, synthetic NT 1-13 is used in *in vitro* studies to determine its effect on islet cells.

ENP can be isolated from mammalian plasma or tissue, particularly mammalian ileum. The isolated peptide can then be sequenced by conventional peptide/protein sequencing techniques. From the ENP amino acid sequence, an ENP oligonucleotide probe can be designed and obtained. This ENP oligonucleotide probe can then be used to further characterize tissue distribution of ENP. For instance, an ENP oligonucleotide probe will permit examination of ENP expression in the brain and further analysis of ENP expression in the gut. The ENP amino acid sequence can also be compared to known amino

acid protein/peptide sequences, to determine ENP's relationship to such known proteins/peptides.

Since the molecular weight of ENP is about 3500 Da (estimated to correspond to about 30-35 amino acids), ENP is amendable to synthetic production. Such synthetically produced ENP, and/or portions thereof, will be useful in generating a panel of reagents (i.e., monoclonal and polyclonal antibodies, oligonucleotide probes, truncated peptides, mutated peptides, chimeric peptides and the like) that may be used to further characterize ENP and its biological/physiological activities. Synthetically produced ENP polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue. For instance, isolated, purified ENP may be added to cell cultures derived from pancreatic or gastrointestinal tissues, to determine ENP's effects on cell differentiation, proliferation and/or metabolism. Labeled ENP may be administered orally or parenterally in an appropriate animal model, and sites of ENP binding may be determined. In tissues or organs where ENP interacts, *in vivo* effects on nutrient metabolism and organ function (for instance, pancreatic islet function) can be examined. ENP can also be used to identify its corresponding receptor. Administration of anti-ENP antibody in an appropriate model system, or generation of ENP knockout models, can also aid in delineating ENP's role *in vivo*.

More specifically, truncated peptides can be generated from altered or unaltered ENP by synthesizing peptides wherein amino acid residues are truncated from the N- or C-terminus to determine the shortest active peptide, or between the N- and C-terminus to determine the shortest active sequence. The physical and biological properties of the native and modified ENPs may be assessed in a number of ways. Mass spectral analysis methods such

as electrospray and Matrix-Assisted Laser Desorption/ Ionization Time Of Flight mass spectrometry (MALDI TOF) analysis are routinely used in the art to provide such information as molecular weight and to confirm disulfide bond formation.

In a prototypical approach, the novel peptide can be assembled on Rink amide MBHA resin using a 432A Applied Biosystems, Inc. (Foster City, CA) automated peptide synthesizer and solid phase strategy. Double coupling may ensure completion of the coupling reaction, and HBtu-HOBt coupling chemistry may be used. Each cycle may include Fmoc deprotection of amine from the amino acid residue on the resin, and coupling of incoming Fmoc-amino acid. After successful assembly of a synthetic ENP, the resin is washed and dried under vacuum for two hours. The peptide resin is resuspended in trifluoroacetic acid (TFA) containing scavengers. This suspension is gently mixed at room temperature, filtered, and the filtrate is collected in an organic solvent mixture. The precipitate is allowed to settle, after which the crude precipitated peptide is isolated.

The crude ENP is then subjected to reverse phase HPLC. The main peak is isolated by preparative reverse phase HPLC using a solvent gradient. Fractions are collected and lyophilized to remove all solvent. Fractions are analyzed by reverse HPLC and the pure fractions are further characterized by mass spectrometry.

From the amino acid sequence of ENP, polynucleotides that encode ENP can be identified and sequenced. An ENP polynucleotide sequence will hybridize to a homologous sequence, or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at

which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is at least about 0.02 M at pH 7 and the temperature is at least about 60°C.

Isolated ENP polynucleotides include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. It is generally preferred to isolate RNA from gastrointestinal or brain tissue, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., Biochemistry 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-1412, 1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. Polynucleotides encoding ENP are then identified and isolated by, for example, hybridization or PCR.

The present invention further provides counterpart polypeptides and polynucleotides from other species (orthologs or paralogs). Of particular interest are ENPs from other mammalian species, including murine, rat, porcine, ovine, bovine, canine, feline, equine and other primate proteins. Species homologs of the human proteins can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses ENP. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from ENP sequences. A library is then prepared from mRNA of a positive tissue of cell line. An ENP-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the ENP polynucleotide and polypeptide sequences. A cDNA can also

be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to ENP. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that a specific ENP sequence represents a single allele of the ENP gene and polypeptide, and that allelic variation and alternative splicing are expected to occur. Allelic variants can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of an ENP DNA sequence include those containing silent mutations and those in which mutations result in amino acid sequence, as well as proteins which are allelic variants of a specific ENP polypeptide sequence.

As used herein, "ENP" includes isolated ENP polypeptides that are substantially homologous to the ENP polypeptide described herein and their species homologs/ orthologs. The term "substantially homologous" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to ENP or its orthologs or paralogs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to ENP or its orthologs or paralogs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-616, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 1 (amino

acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 2) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), maltose binding protein (Kellerman and Ferenci, Methods Enzymol. 90:459-463, 1982; Guan et al., Gene 67:21-30, 1987), or other antigenic epitope or binding domain. See, in general, Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA).

Table 2

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline and α-methyl serine) may be substituted for ENP amino acid residues. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for ENP amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, or preferably, are commercially available, and include pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

Essential amino acids in ENP of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-1085, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., tissue or receptor binding, fat intake feedback, satiety effect and the like) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-4708, 1996. Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related enteric/brain peptides or proteins.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-56, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-37, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204)

and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Mutagenesis methods as disclosed above can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active polypeptides can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure. Using the methods discussed above, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially homologous to ENP and retain the functional and biological properties of the wild-type protein. Such polypeptides may include additional polypeptide segments as generally disclosed above.

ENPs of the present invention, including full-length peptides, fragments thereof and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987, which are incorporated herein by reference.

In general, a DNA sequence encoding an ENP polypeptide of the present invention is operably linked

to other genetic elements required for its expression, generally including a transcription promoter and terminator within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct an ENP polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be naturally associated with native ENP, pro-ENP or other ENP precursor, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is joined to the ENP DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells are also preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981: Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated

transfection (Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), liposome-mediated transfection (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993), and viral vectors, which are incorporated herein by reference. The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference. Preferred cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978, which are incorporated herein by reference) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as

"amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222; Bang et al., U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463, which are incorporated herein by reference. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987.

Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing ENPs, fragments or polypeptide fusions thereof. Methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075, which are incorporated herein by reference. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed

cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092, which are incorporated herein by reference) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454, which are incorporated herein by reference. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-65, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228, which is incorporated herein by reference. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533, which is incorporated herein by reference.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on

the expression vector or co-transfected into the host cell.

Expressed recombinant ENP polypeptides (or chimeric ENP polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ) being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art.

Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can be isolated by exploitation of their biochemical properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins. Briefly, a gel is first charged with divalent metal ions to form a chelate (E. Sulkowski, Trends in Biochem. 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39). Alternatively, a fusion of the polypeptide of interest and an affinity tag (e.g., polyhistidine, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

The activity of molecules of the present invention can be measured using a variety of assays that measure gut/brain peptide activity. Of particular interest are changes in glucose, insulin, free fatty acid, NPY and ob protein production and/or metabolism. Such assays are well known in the art.

In view of the tissue distribution observed for ENP, agonists and antagonists have enormous potential in both *in vitro* and *in vivo* applications. Compounds identified as ENP agonists may be useful as satiety agents and/or to control fat intake *in vivo*. Agonist compounds may also be useful as components of defined

cell culture media, and may be used alone or in combination with other cytokines and hormones to replace serum that is commonly used in cell culture.

ENP antagonists may be used in conditions where appetite and fat intake are beneficially up-regulated (i.e., anorexia and other eating disorders, cachexia, radiation and chemotherapy appetite depression and the like). ENP agonists and antagonists may also prove useful in the study of ENP-directed gastro- and neuromodulation. Antagonists are also useful as research reagents for characterizing sites of ENP-receptor interaction.

ENP can also be used for purification of ENP-binding moieties (such as ENP receptor). The ENP polypeptide is immobilized on a solid support, such as beads of agarose, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-linked polyacrylamide, or like materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide activation. The resulting medium will generally be configured in the form of a column, and fluids containing ligand-binding moiety are passed through the column one or more times to allow a ligand-binding moiety to bind to the ENP polypeptide. The ligand-binding moiety is then eluted using changes in salt concentration, chaotropic agents (guanidine HCl), or pH to disrupt ligand-receptor binding.

An assay system that uses an ENP-binding receptor (or an antibody, one member of a complement/anti-complement pair) or a binding fragment thereof, and a commercially available biosensor instrument (BIAcore™, Pharmacia Biosensor, Piscataway, NJ) may be advantageously employed. Such receptor, antibody, member of a complement/anti-complement pair or fragment is

immobilized onto the surface of a receptor chip. Use of this instrument is disclosed by Karlsson, J. Immunol. Methods 145:229-40, 1991 and Cunningham and Wells, J. Mol. Biol. 234:554-63, 1993. A receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding.

ENP-binding polypeptides can also be used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity (see Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949) and calorimetric assays (Cunningham et al., Science 253:545-48, 1991; Cunningham et al., Science 245:821-25, 1991).

ENP polypeptides can also be used to prepare antibodies that specifically bind to ENP epitopes, peptides or polypeptides. Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982, which are incorporated herein by reference). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals, such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats.

The immunogenicity of an ENP polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of ENP or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as $F(ab')_2$ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to ENP protein or peptide, and selection of antibody display libraries in phage or

similar vectors (for instance, through use of immobilized or labeled ENP protein or peptide).

Antibodies are defined to be specifically binding if they bind to a ENP polypeptide with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art (for example, by Scatchard analysis).

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to ENP proteins or peptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant ENP protein or peptide.

Antibodies to ENP may be used for tagging cells that express ENP; for isolating ENP by affinity purification; for diagnostic assays for determining circulating levels of ENP polypeptides; for detecting or quantitating soluble ENP as marker of underlying pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block ENP biological or physiological activity *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as

intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications.

Molecules of the present invention can be used to identify and isolate receptors involved in satiety and fat intake. For example, proteins and peptides of the present invention can be immobilized on a column and membrane preparations run over the column (Immobilized Affinity Ligand Techniques, Hermanson et al., eds., Academic Press, San Diego, CA, 1992, pp.195-202). Proteins and peptides can also be radiolabeled (Methods in Enzymol., vol. 182, "Guide to Protein Purification", M. Deutscher, ed., Acad. Press, San Diego, 1990, 721-737) or photoaffinity labeled (Brunner et al., Ann. Rev. Biochem. 62:483-514, 1993 and Fedan et al., Biochem. Pharmacol. 33:1167-80, 1984) and specific cell-surface proteins can be identified.

The ENPS of the present invention will be useful in treatment or modulation of satiety/obesity disorders, diabetes (particularly Type II diabetes) and/or digestive disorders. The ENPs of the present invention may used to modulate or to treat or prevent development of pathological conditions in such diverse tissue as gastrointestinal or brain tissue.

For pharmaceutical use, the peptides of the present invention are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include an ENP protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial

surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington's Pharmaceutical Sciences, Gennaro, ed., Mack Publishing Co., Easton PA, 1990, which is incorporated herein by reference. Therapeutic doses will generally be in the range of 0.1 to 100 lg/kg of patient weight per day, preferably 0.5-20 lg/kg per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years. In general, a therapeutically effective amount of an ENP is an amount sufficient to produce a clinically significant change in appetite, fat intake or diabetic symptoms.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1. Immunoidentification of ENP

Prosomatostatin (also designated Pro-S, somatostatin-28 or S-28) is a precursor polypeptide that is processed to somatostatin-14 (the C-terminal 14 amino acids of prosomatostatin). Several anti-Pro-S antisera were prepared by immunizing animals with synthetic peptides spanning different regions of Pro-S. One antiserum, designated F4, reacted with Asn(5)-Pro(6)-Ala(7) residues in the N-terminal domain of Pro-S.

For immunosorption of plasma and tissue extracts, F-4 was coupled to agarose. After passage of a biological sample through the immunoadsorbent matrix, the F-4-bound material was eluted from the matrix by

acidification (pH 2-3) ("F-4 eluate"). To measure S-28 levels in the F-4 eluate, a second, anti-Pro-S antiserum, directed against a C-terminal epitope of Pro-S, was used in an RIA format ("C-terminal RIA"). However, if the F-4 eluate was measured in an RIA format using F-4 as the detecting antibody ("F-4 RIA"), the level of material detected was 10 times greater than when a C-terminal RIA was used to directly measure S-28. When F-4 eluate was passed through a Biogel P-10 column at pH 8.5, an F-4 immunoreactive peak eluted prior to S-28. This peak material was designated "5K' or "ENP" (enteric neural peptide). Levels of ENP increased 2-3 fold within 30 min after intake of mixed food, wherein fat was the predominant nutrient signal. ENP did not cross-react with any other anti-Pro-S antisera directed against C- or N-terminal synthetic peptides of Pro-S, or with antisera directed against other known, enteric neural peptides.

Example 2. Tissue Distribution

Following tissue extraction in 2 N HAc, the highest concentrations of ENP were detected in the mammalian distal ileum. By immunocytochemistry using F-4 antiserum, neuron cell bodies within the myenteric plexus and neuronal axons ramifying into the mucosa were detected in the ileum. These tissue sites did not stain when other anti-Pro-S antibodies were used.

Example 3. ENP Isolation

ENP was isolated from monkey ileum by acid extraction, gel filtration, affinity chromatography and HPLC. By gel filtration in 6 M guanidine, the peptide exhibited an apparent molecular weight of 3500 Da. By epitope analysis, ENP did not coincide with the sequence of any reported enteral neural peptide.

Example 4. ENP Analysis

ENP was fractionated using a variety of HPLC techniques. HPLC fractionation was improved with the addition of an ion exchange chromatography step after affinity purification. Amino acid sequencing and MALDI analysis of semi-pure fractions indicated that ENP corresponds to the first 13 residues of somatostatin-28 (i.e, N-terminal 1-13 or NT 1-13). More specifically, the sequence data showed the following:

	sxNxNPAMAxxEx	(SEQ ID NO. 1)
NT 1-13	SANSNPAMAPRER	(SEQ ID NO. 2)

MALDI data showed a mass consistent with the mass of NT 1-13 having an oxidized Met (1416 Da). The post-source decay (PSD) spectrum had about 15 fragment ion peaks that were consistent with a reference PSD spectrum of synthetic NT 1-13 ox. Synthetic NT 1-13 displayed a higher affinity than S1-28 for the F4 polyclonal antiserum.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: University of Washington
Seattle
WA
98195
USA

ZymoGenetics, Inc.
1201 Eastlake Avenue East
Seattle
WA
USA
98102

(ii) TITLE OF THE INVENTION: ENTERIC NEURAL PEPTIDE

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ZymoGenetics, Inc.
(B) STREET: 1201 Eastlake Avenue East
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 98102

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Leith, Debra K
(B) REGISTRATION NUMBER: 32,619
(C) REFERENCE/DOCKET NUMBER: 96-19PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-442-6674
(B) TELEFAX: 206-442-6678
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Xaa Asn Xaa Asn Pro Ala Met Ala Xaa Xaa Glu Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Ala Asn Ser Asn Pro Ala Met Ala Pro Arg Glu Arg
1 5 10

CLAIMS

1. A method for regulating obesity or an eating disorder in a mammal, comprising:

administering to a mammal in need thereof a composition comprising a mammalian polypeptide having 90% amino acid sequence identity to SEQ ID NO. 2 in combination with a pharmaceutically acceptable vehicle in an amount sufficient to regulate obesity or the eating disorder.

2. The method of claim 1 wherein the polypeptide has the amino acid sequence SANSNPAMAPRER.

3. A method for treating diabetes in a mammal, comprising:

administering to a mammal in need thereof a composition comprising a mammalian polypeptide having 90% amino acid sequence identity to SEQ ID NO. 2 in combination with a pharmaceutically acceptable vehicle in an amount sufficient to treat diabetes.

4. The method of claim 3 wherein the polypeptide has the amino acid sequence SANSNPAMAPRER.

5. An antibody that binds to Asn(5)-Pro(6)-Ala(7) of prosomatostatin.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/655, 7/08, A61K 38/31, 38/10, C07K 16/26

A3

(11) International Publication Number: WO 98/09991

(43) International Publication Date: 12 March 1998 (12.03.98)

(21) International Application Number: PCT/US97/15578

(22) International Filing Date: 4 September 1997 (04.09.97)

(30) Priority Data:
60/025,468 5 September 1996 (05.09.96) US

(71) Applicants: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). UNIVERSITY OF WASHINGTON [US/US]; Seattle, WA 98195 (US).

(72) Inventors: ENSINCK, John, W.; 232 Dorffel Drive East, Seattle, WA 98112 (US). VOGEL, Robin, E.; 9230 S.W. 192nd Street, Vashon, WA 98070 (US). LASCHANSKY, Ellen, C.; 2229 11th Avenue East, Seattle, WA 98102 (US). FRANCIS, Bruce, H.; 1660 Interlaken Place East, Seattle, WA 98112 (US). BASKIN, Denis, G.; 6205 145th Avenue N.E., Redmond, WA 98052 (US). D'ALESSIO, David, A.; 4607 Woodland Park Avenue North, Seattle, WA 98103 (US). HOFFMAN, Ross, C.; Apartment 302, 503 13th Avenue East, Seattle, WA 98102 (US).

(74) Agent: LEITH, Debra, K.; ZymoGenetics, Inc., Director, Intellectual Property & Licensing, 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
16 July 1998 (16.07.98)

(54) Title: ENTERIC NEURAL PEPTIDE

(57) Abstract

The present invention relates to an intestinal peptide that is located in mammalian distal ileum. The peptide, designated "enteric neural peptide" or "ENP", corresponds to the N-terminal, 13 amino acid fragment of somatostatin-28. ENP is localized in cells that do not express S-28 and do not contain the C-terminal S-14 fragment of prosomatostatin. ENP increases in the circulation within 30 minutes of intake of mixed food, wherein the predominant nutrient signal is fat. Antibodies that bind ENP are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inter[illegible]Application No
PCT/US 97/15578

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/655 C07K7/08 A61K38/31 A61K38/10 C07K16/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BASKIN, D. G. & ENSINCK, J.W.: "Somatostatin in epithelial cells ..." PEPTIDES, vol. 5, 1984, pages 615-621, XP002050446 * whole disclosure * ---	1,2
A	KODA, L.Y. ET AL.: "Blood pressure following microinjection of somatostatin related peptides ..." EUR. J. PHARMACOL., vol. 113, 1985, pages 425-430, XP002050447 * whole disclosure * -----	1,2

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search
17 December 1997

Date of mailing of the international search report
15. 05. 98

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer
HERMANN R.

Form PCT/ISA/210 (second sheet) (July 1992)